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PATHOGENESIS AND IMMUNITY

Infection and Replication of Influenza Virus at the Ocular Surface

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ABSTRACT Although influenza viruses typically cause respiratory tract disease, some viruses, particularly those with an H7 hemagglutinin, have been isolated from the eyes of conjunctivitis cases. Previous work has shown that isolates of multiple subtypes from both ocular and respiratory infections are capable of replication in human *ex vivo* ocular tissues and corneal or conjunctival cell monolayers, leaving the determinants of ocular tropism unclear. Here, we evaluated the effect of several variables on tropism for ocular cells cultured *in vitro* and examined the potential effect of the tear film on viral infectivity. All viruses tested were able to replicate in primary human corneal epithelial cell monolayers subjected to aerosol inoculation. The temperature at which cells were cultured postinoculation minimally affected infectivity. Replication efficiency, in contrast, was reduced at 33°C relative to that at 37°C, and this effect was slightly greater for the conjunctivitis isolates than for the respiratory ones. With the exception of a seasonal H3N2 virus, the subset of viruses studied in multilayer corneal tissue constructs also replicated productively after either aerosol or liquid inoculation. Human tears significantly inhibited the hemagglutination of both ocular and nonocular isolates, but the effect on viral infectivity was more variable, with tears reducing the infectivity of nonocular isolates more than ocular isolates. These data suggest that most influenza viruses may be capable of establishing infection if they reach the surface of ocular cells but that this is more likely for ocular-tropic viruses, as they are better able to maintain their infectivity during passage through the tear film.

IMPORTANCE The potential spread of zoonotic influenza viruses to humans represents an important threat to public health. Unfortunately, despite the importance of cellular and tissue tropism to pathogenesis, determinants of influenza virus tropism have yet to be fully elucidated. Here, we sought to identify factors that limit the ability of most influenza viruses to cause ocular infection. Although ocular symptoms in humans caused by avian influenza viruses tend to be relatively mild, these infections are concerning due to the potential of the ocular surface to serve as a portal of entry for viruses that go on to establish respiratory infections. Furthermore, a better understanding of the factors that influence infection and replication in this noncanonical site may point toward novel determinants of tropism in the respiratory tract.

KEYWORDS influenza, eye, ocular, tropism, aerosol, tears, tear film, conjunctivitis

In contrast to other influenza A viruses, which rarely cause ocular symptoms, H7 subtype viruses have historically shown a unique propensity to infect the eye, typically resulting in conjunctivitis. In fact, prior to 2013, the majority of human infections with H7 viruses presented with conjunctivitis, sometimes accompanied by mild respiratory symptoms, suggesting that the H7 hemagglutinin may be an important

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TABLE 1 Influenza viruses used in this study

Virus	Abbreviation	Subtype	Presentation in human case	Reference(s)
A/Panama/2007/1999	Pan/99	H3N2	Seasonal infection	62
A/Mexico/4482/2009	Mex/4482	Pandemic H1N1	Nonfatal respiratory infection	51
A/New York/107/2003	NY/107	LPAI H7N2	Nonfatal respiratory infection	63, 64
A/Netherlands/219/2003	NL/219	HPAI H7N7	Fatal respiratory infection	19, 30
A/Netherlands/230/2003	NL/230	HPAI H7N7	Conjunctivitis	19, 30
A/Mexico/InDRE7218/2012	Mex/7218	HPAI H7N3	Conjunctivitis	65
A/Anhui/1/2013	Anh/1	LPAI H7N9	Fatal respiratory infection	66
A/Italy/3/2013	Italy/3	HPAI H7N7	Conjunctivitis	67
A/Thailand/16/2004	Thai/16	HPAI H5N1	Fatal respiratory infection	52
A/Bangladesh/5487/2011	Bang/5487	HPAI H5N1	Nonfatal respiratory infection	68

determinant of ocular tropism. However, there was a notable absence of conjunctivitis cases during the first wave of human infections with H7N9 viruses in China, and to our knowledge, there have been no subsequent reports of ocular symptoms associated with any of the over 1,500 cases reported to date (1–3). This indicates that the presence of an H7 hemagglutinin may be required for ocular tropism but is not sufficient to confer this property. At this time, the factors that enable certain H7 viruses to infect and replicate in the eye remain unclear.

Neither receptor binding specificity nor protease susceptibility, two major determinants of influenza virus tropism, can currently explain why such a limited set of viruses appears capable of causing ocular infections, although the eyes are presumably exposed to similar fomites, respiratory droplets, and aerosols as is the respiratory tract. With the exception of highly pathogenic avian strains, influenza viruses have traditionally been thought to be limited to the respiratory and intestinal tracts due to their dependence on host proteases present only in these tissues for hemagglutinin cleavage (4). However, the fact that low-pathogenic avian influenza (LPAI) viruses have been associated with conjunctivitis in humans and cause disease at rates comparable to those of highly pathogenic avian influenza (HPAI) viruses following ocular inoculation in mammalian models suggests that proteases present in the human eye are capable of performing this function (5–7). Similarly, the predominance of α2,3-linked sialic acid (SA) on the ocular surface may explain the poor susceptibility to human influenza viruses, which preferentially bind α2,6-linked glycans, but not to avian H5 or H9 viruses (8–10).

Previous studies have shown that influenza A viruses of multiple subtypes can replicate in various human ocular cells and *ex vivo* tissues (9, 11–13). This suggests that tropism may be affected by cellular characteristics, such as mucin expression, that differ between cells present in the eye and those cultured in the laboratory. Alternatively, tropism for ocular tissue may be governed not by cellular susceptibility and permissiveness but by conditions external to cells themselves. We considered both possibilities in this study: in addition to examining how viral replication in corneal epithelial cells was affected by inoculation method, temperature, and culture complexity, we also investigated the interaction between influenza viruses and human tears. Our data ultimately supported the latter hypothesis, indicating that the ability to retain infectivity during passage through the tear film may determine which viruses are able to establish infection of the eye.

RESULTS

Ocular and nonocular influenza viruses are capable of replication in primary human corneal epithelial cells. To confirm previous findings in our experimental system, we first evaluated the ability of a panel of five influenza A viruses (Table 1) to replicate in primary human corneal epithelial cells. The panel included representative HPAI viruses isolated from ocular swabs of human conjunctivitis cases associated with poultry outbreaks in The Netherlands (A/Netherlands/230/2003 [NL/230]) and Mexico (A/Mexico/InDRE7218/2012 [Mex/7218]) in 2003 and 2012, respectively. Both viruses are described here as ocular or ocular-tropic. For comparison, we used a virus from one

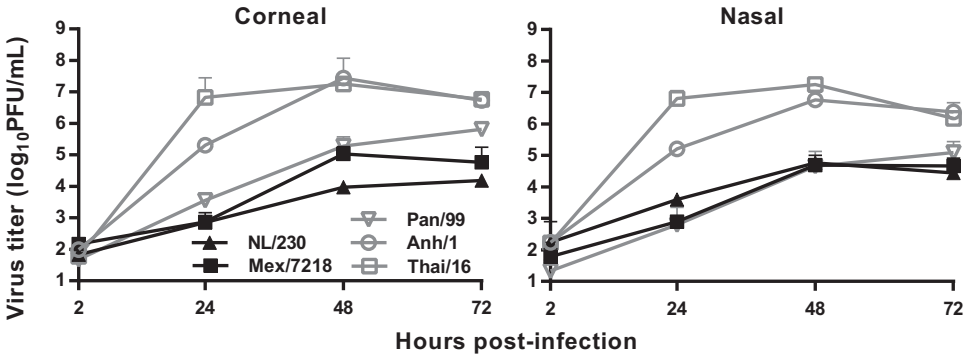

FIG 1 Influenza virus replication in primary human corneal and nasal cell monolayers after liquid inoculation. Cells (culture area, 4.67 cm^2) were infected with ocular (shown in black) and nonocular (shown in gray) viruses at an MOI of 0.01. Titers in the supernatants collected at the indicated times were determined by plaque assay. Each point represents the mean of results from three replicates, with error bars indicating standard deviations. The limit of detection was 1 log_{10} PFU/ml.

of the early H7N9 cases in China (A/Anhui/1/2013 [Anh/1]), an HPAI H5N1 virus (A/Thailand/16/2004 [Thai/16]), and a seasonal H3N2 virus (A/Panama/2007/1999 [Pan/99]). These viruses were isolated from the respiratory tracts of human cases that displayed no ocular symptoms and are considered respiratory or non-ocular-tropic. In accordance with data in the literature, all viruses tested were able to replicate productively, reaching titers of at least 10^4 PFU/ml following inoculation at a multiplicity of infection (MOI) of 0.01 (Fig. 1). In fact, the respiratory-tropic viruses replicated more efficiently than did the ocular-tropic ones, underscoring the poor correlation between *in vivo* tropism and the replication kinetics seen in this *in vitro* model. A similar pattern was observed in parallel experiments in primary human nasal epithelial cells (Fig. 1), suggesting that the superior ability of the respiratory viruses to reach high titers in corneal cells reflects underlying differences in the replicative capacities of the viruses tested (for example, the presence of a lysine at position 627 in the PB2 gene) rather than cell type-specific effects (14).

Inoculation method and culture temperature do not alter tropism for corneal cells *in vitro*. Although some persons experiencing influenza-associated conjunctivitis report trauma to the eye (6, 15), for most, the only known exposure is contact with infected poultry, often during culling operations, for which eye protection is recommended but not necessarily worn (16, 17). This suggests that ocular infection usually occurs either from workers touching their eyes with contaminated hands or from the virus traveling through the air. We hypothesized that ocular viruses might be better able than others to infect ocular cells via aerosol or might replicate more efficiently at the sub-37°C temperature of the human ocular surface. To determine if this was the case, we exposed corneal cell monolayers to four different aerosol doses of the viruses examined previously. After simultaneous inoculation, half of the culture wells were incubated at 37°C, and the other half were incubated at 33°C (Fig. 2).

As with traditional liquid-based inoculation, all viruses were able to replicate productively after aerosol inoculation. This was true even for low doses of the virus. For cells cultured at 37°C, 50% infectious doses (ID_{50}s) were $\leq$10 PFU (Fig. 2). At 33°C, ID_{50}s were similar, except in the case of the NL/230 virus, which had an ID_{50} of 83 PFU at 33°C, compared to 10 PFU at 37°C. Consistent with data for respiratory cells (18), temperature had little effect on the growth of the seasonal virus Pan/99, whereas the growth of the avian viruses was attenuated at the lower temperature. The effects of temperature on the curve shape were similar across inoculation doses, but differences in peak titers were greater at lower exposure doses, possibly because the low dosage delayed replication at both temperatures such that titers had not plateaued by 96 h postinfection.

In order to confirm that our findings were not specific to individual virus strains but could be applied to groups of similar isolates, we expanded our panel of viruses

A

NL/230						
Exposure dose	37°C			33°C		
	1	2	3	1	2	3
9300	+	+	+	+	+	+
708	+	+	+	+	+	+
40	-	+	+	-	-	+
2.5	-	+	-	-	-	-
ID_{50}	10			83		
CID_{50}	$3.7x10^{-6}$			$1.0x10^{-4}$		

Mex/7218						
Exposure dose	37°C			33°C		
	1	2	3	1	2	3
5863	+	+	+	+	+	+
526	+	+	+	+	+	+
15	+	+	+	-	+	+
1.2	+	+	+	-	-	-
ID_{50}	≤3.0			8.0		
CID_{50}	$≤3.7x10^{-6}$			$9.7x10^{-6}$		

Anh/1						
Exposure dose	37°C			33°C		
	1	2	3	1	2	3
2224	+	+	+	+	+	+
283	+	+	+	+	+	+
16	+	+	+	+	+	+
1.2	-	-	-	-	-	-
ID_{50}	4.4			4.4		
CID_{50}	$9.5x10^{-6}$			$9.5x10^{-6}$		

Thai/16						
Exposure dose	37°C			33°C		
	1	2	3	1	2	3
2608	+	+	+	+	+	+
384	+	+	+	+	+	+
9.7	+	+	+	+	-	+
2.0	-	-	-	-	-	-
ID_{50}	4.4			6.6		
CID_{50}	$9.5x10^{-6}$			$1.4x10^{-5}$		

Pan/99						
Exposure dose	37°C			33°C		
	1	2	3	1	2	3
4448	+	+	+	+	+	+
1152	+	+	+	+	+	+
13	-	+	+	+	+	+
4.3	-	-	-	-	-	-
ID_{50}	9.6			7.3		
CID_{50}	$2.1x10^{-5}$			$1.6x10^{-5}$		

B

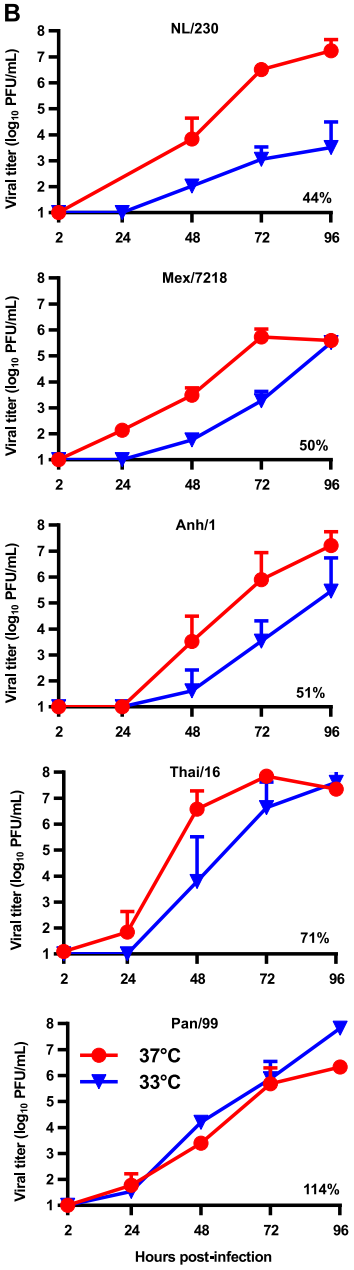

FIG 2 Infectivity and replication in primary human corneal cell monolayers after aerosol inoculation. (A) Triplicate wells (4.67 cm^2) were exposed to the indicated viruses at the doses (in PFU) shown. + indicates wells for which infectious virus was detected in the culture supernatant by plaque assay by 96 h postinfection (limit of detection, 10 PFU/ml). The CID_{50} (50% cellular infectious dose) is given in PFU per cell and represents the MOI associated with the ID_{50}. (B) Replication curves for the rows shown in boldface type in panel A. Means and standard deviations are shown. Numbers at the bottom right of each graph indicate the area under the replication curve at 33°C as a percentage of the area under the curve at 37°C.

(Table 1) to include an additional HPAI H7N7 ocular virus, A/Italy/3/2013 (Italy/3), isolated from the eye of a poultry worker with conjunctivitis, and three respiratory viruses: the early pandemic H1N1 isolate A/Mexico/4482/2009 (Mex/4482), an isolate from a single human LPAI H7N2 respiratory case that occurred in New York in 2003 (A/New York/107/2003 [NY/107]), and an additional human HPAI H5N1 isolate (A/Bangladesh/5487/2011 [Bang/5487]). The A/Netherlands/219/2003 (NL/219) virus, which was isolated from the lone fatal human case associated with the same 2003 Netherlands conjunctivitis outbreak as the NL/230 virus and differs from NL/230 by 15

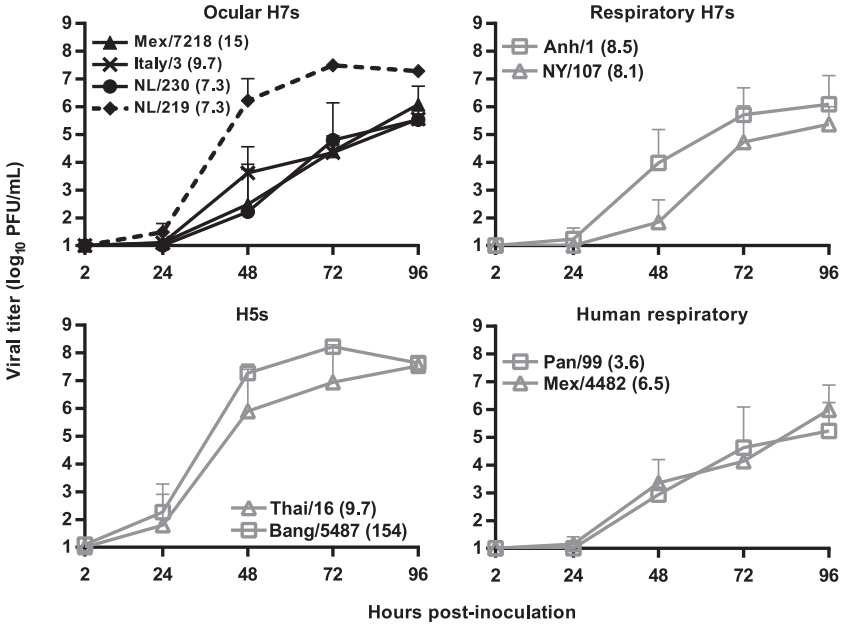

FIG 3 Replication kinetics in primary human corneal cell monolayers infected by low-dose aerosolized virus. The growth curve for NL/219 is dashed to highlight the unique nature of this isolate. The NL/230 virus infected only 2/3 wells, and the mean for the two infected wells is shown. For all other viruses, each point represents the mean of results from three independent wells and the standard deviation. Exact exposure doses in PFU/well are listed after virus names. The limit of detection was 1 $\log_{10}$ PFU/ml.

amino acids, including position 627 of PB2 (lysine in NL/219 versus glutamic acid in NL/230), was also included. Although the case patient suffered from chronic blepharitis and may have been infected as a result of ocular exposure, his symptoms were exclusively respiratory, and the virus was obtained from a postmortem lung specimen (19). Nonetheless, when grouping was necessary, we categorized NL/219 as an ocular virus on the basis of its isolation as part of an outbreak strongly associated with human ocular disease and its genotypic and phenotypic similarities to ocular viruses (9, 12). A single, low inoculation dose (target dose of ~10 PFU) was used. Inoculations occurred via the aerosol route, and all cells, sourced from a different donor than the one in the previous experiment, were cultured at 37°C postinoculation. Again, all viruses tested were able to establish infection and replicate, ultimately reaching titers of at least 10^5 PFU/ml (Fig. 3). Replication curves for Pan/99 (seasonal H3N2) and Mex/4482 (pandemic H1N1) viruses were nearly identical, with a steady increase in the viral titer between 24 and 96 h after infection. The two respiratory H7 subtype viruses showed similar growth kinetics, although the titers of the Anh/1 virus were approximately 10 times higher than those of the NY/107 virus. In contrast, the two H5N1 viruses grew more rapidly early after inoculation, with titers plateauing after 48 h. The growth kinetics of the three ocular viruses were nearly identical, peaking at approximately the same titer ($10^{5.5}$ PFU/ml). The growth of the NL/219 virus was similar to that of the H5N1 viruses, with a maximum titer that was 100-fold higher than that of the H7 ocular isolates. In summary, neither the inoculation method nor the culture temperature restricted the ability of respiratory isolates to productively infect human corneal epithelial cells.

Viral infection of corneal tissue constructs. While cryopreserved cells grown in monolayers are a useful and flexible research tool, there are many features of the ocular surface that are potentially relevant to viral infection but are not recreated by typical culture methods. We next examined commercially available corneal tissue constructs (EpiCorneal; MatTek) to determine whether these constructs might provide an enhanced *in vitro* model of ocular influenza virus infection. The tissue constructs comprise a stratified epithelium with approximately five layers of cells and are grown at the

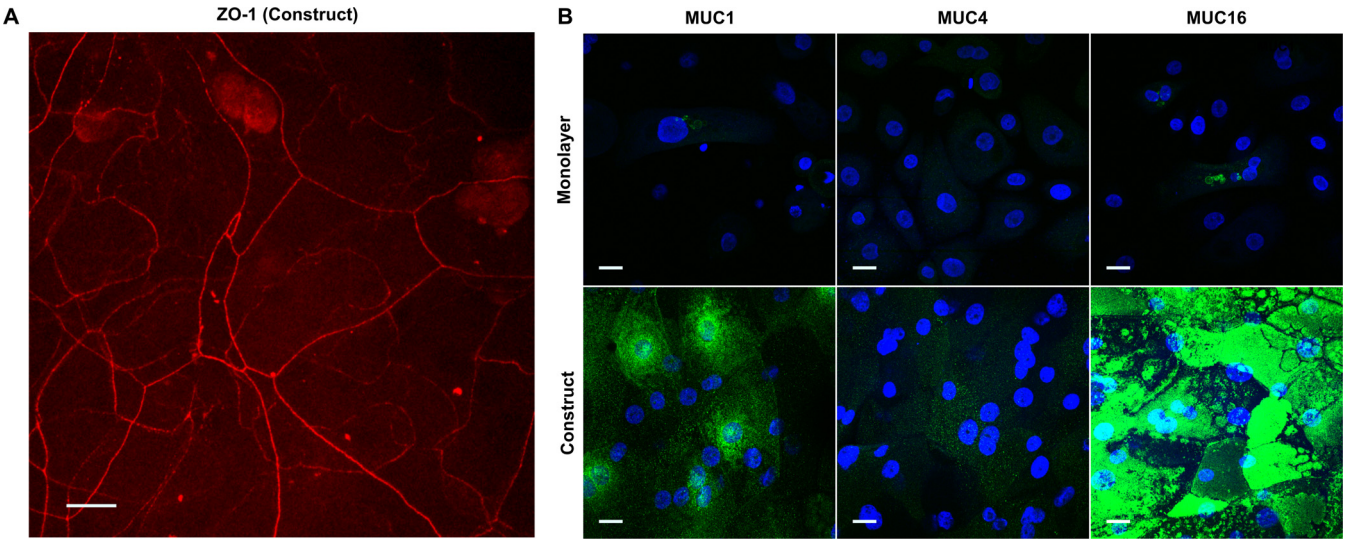

FIG 4 Tight junctions and mucin expression at the apical surface of primary corneal cell monolayers and corneal tissue constructs. (A) Constructs were fixed and stained with antibody to the tight junction protein ZO-1 (red). Bar = 20 μm. (B) Uninfected constructs and monolayers were fixed and stained with DAPI (4′,6-diamidino-2-phenylindole) (blue) and monoclonal antibodies against MUC1, MUC4, or MUC16 (green). Images are maximum-intensity projections across 8.28 μm of the *z* axis. Bars = 20 μm.

air-liquid interface. While low transepithelial resistance indicated that the corneal cell monolayers described above lacked tight junctions, these tight junctions were present in the apical layer of the constructs, as shown by positive ZO-1 staining (Fig. 4A). Additionally, the expression levels of the major tethered corneal mucins, MUC1, MUC4, and MUC16, were higher in the constructs than in the cell monolayers (Fig. 4B). Staining with *Sambucus nigra* lectin (SNA) and *Maackia amurensis* lectin I (MAA-I) indicated that both α2,6- and α2,3-linked sialic acids were present on the apical surface of both monolayers and constructs, although, consistent with human *ex vivo* tissue staining of the conjunctiva, α2,3 linkages were more prevalent (Fig. 5) (8). MAA-II was not included, as little to no binding was observed in a pilot experiment. In contrast to the respiratory cell line Calu-3, in which the two lectins stain distinct cells, many of the corneal cells were bound by both lectins, suggesting the coexpression of the two types of sialic acid receptors.

Although we had found that a variety of viruses could infect and replicate in corneal cell monolayers after aerosol inoculation, we suspected that the more extensive mucin layer and greater differentiation of cells in the constructs might inhibit infection by some influenza viruses more than others. We therefore exposed constructs to one of three different doses of aerosolized NL/230, Anh/1, Thai/16, or Pan/99 virus or to a liquid inoculum at an MOI of 0.01. Infection and replication were limited at the lowest aerosol dose ($\leq$5 PFU), with only the NL/230 virus reaching titers above 100 PFU and only in 2/3 replicates (Fig. 6). At higher doses, viral replication appeared to be productive for NL/230, Anh/1, and Thai/16 viruses, with titers increasing over time, particularly in the first 48 h. Limited production of the Pan/99 virus was seen in the liquid-inoculated wells, whereas the titer in the aerosol-inoculated ones either did not increase or dropped to undetectable levels at 48 h postinfection.

Given that both aerosol and liquid inocula reliably infected cells, liquid-based inoculation was used for all further experiments in order to reduce variability in the multiplicity of infection. First, we generated replication curves for four additional viruses in the corneal constructs at an MOI of 0.01. Mex/7218 and NL/219 viruses replicated most efficiently, with the growth curve of the NL/219 virus resembling that of Thai/16, although, unlike Thai/16, it plateaued by 48 h (Fig. 7A). Unlike Anh/1, an additional H7 respiratory virus (NY/107) replicated relatively poorly, with titers being similar to those of the seasonal Pan/99 virus. Mex/4482, a pandemic H1N1 virus, grew

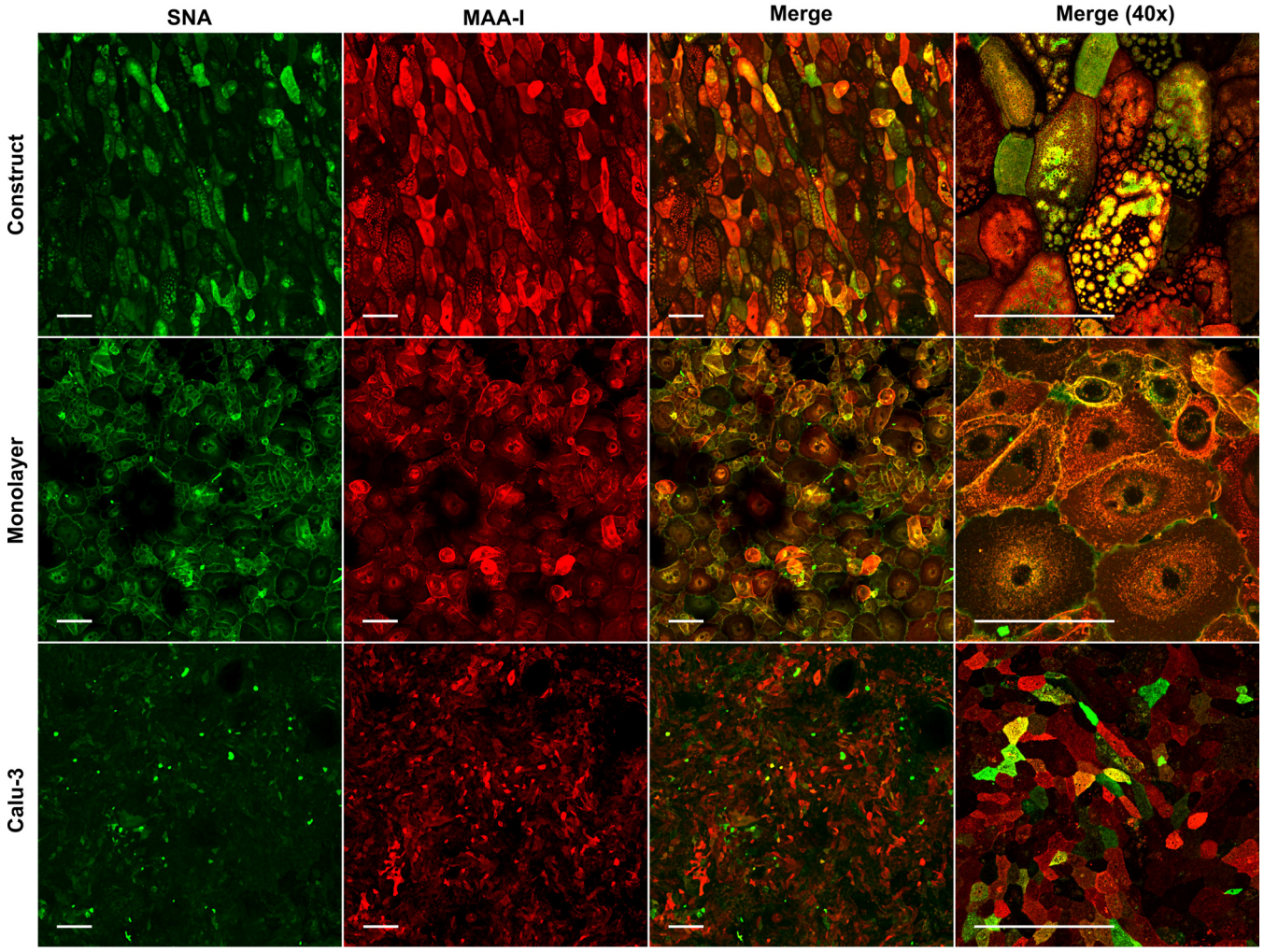

FIG 5 α2,6- and α2,3-linked sialic acid expression on corneal cell monolayers and tissue constructs. The bronchial epithelial cell line Calu-3 is included for comparison. Cells were stained for α2,6-linked sialic acid by using SNA (green) and for α2,3-linked sialic acid by using MAA-I (red). Images are maximum-intensity projections. Bars = 100 μm.

slightly better, with supernatant concentrations at 96 h postinfection being approximately 10 times higher than those of the Pan/99 and NY/107 viruses. In order to evaluate the spread of infection within the construct, we fluorescently stained constructs infected with each virus at 96 h postinfection using an antibody against the viral nucleoprotein (NP). Horizontal spread appeared to be relatively localized, with concentrations of NP-positive (NP^+) cells varying throughout the monolayer. We had anticipated that cells deeper in the stratified epithelium might become infected as the viruses spread vertically but instead found that viral nucleoprotein was generally limited to the apical layer of cells (Fig. 7B).

To compare the abilities of different influenza viruses to initiate infection, we inoculated the constructs at an MOI of 2, fixed the cells 8 h later, and fluorescently stained the viral NP (Fig. 8A). We quantified the level of infection by both counting cells and measuring the mean fluorescence activity per 10$\times$ field. We found significantly more NP^+ cells and more intense cytoplasmic staining around positive nuclei in constructs inoculated with an ocular H7 subtype virus than in those inoculated with a respiratory H7 virus regardless of whether NL/219 was grouped with the ocular viruses or excluded from the analysis (Fig. 8B). Differences in infectivity were much more drastic when we compared the seasonal H3N2 (Pan/99) and pandemic H1N1 (Mex/4482) isolates (Fig. 8C). An average of only 6.3 nuclei per field were positive for Pan/99,

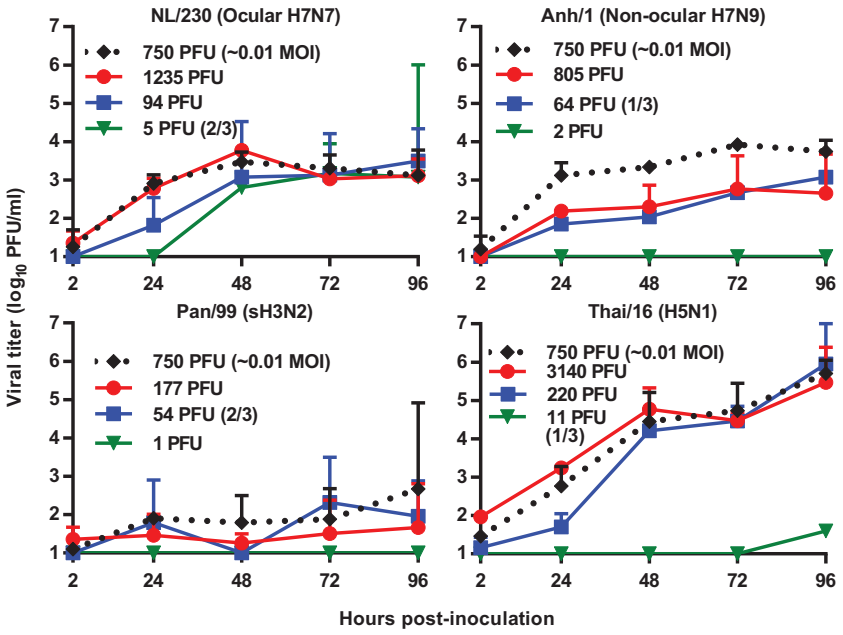

FIG 6 Replication kinetics in corneal tissue constructs. Constructs (0.62 cm^2) were exposed to the indicated amounts of virus via a liquid (dotted line) or aerosol (solid lines) inoculum. The apical surface of each construct was washed with 200 μl medium at the indicated times postinfection, and the viral titer was determined by standard plaque assay. Means and standard deviations ($n = 3$) are shown. The limit of detection was 1 $\log_{10}$ PFU/ml.

compared to 73 for the Mex/4482 virus ($P = 0.013$). As with the nonocular H7 viruses, Pan/99 staining was concentrated, presumably in the nucleus, whereas the Mex/4482 staining pattern was closer to that seen with the ocular H7 viruses. These data indicate that ocular-tropic viruses infect eye cells more efficiently than do non-ocular-tropic viruses.

Ocular viruses are less inhibited by human tears than are nonocular viruses. Having examined the infection efficiencies of viruses directly exposed to ocular cell surfaces, we next examined how the tear film might further amplify the increased probability of an ocular-tropic virus infecting the eye. A thin (7- to 8-μm-thick) layer comprised of lipid, aqueous, and mucus components, the tear film is known to play an important role in protecting the eye from microbial pathogens (20, 21). Because direct sampling of the tear film yields a very minimal volume (the tear volume in the eye is approximately 7 μl, only about 2 μl of which can be collected without stimulating tearing [20]), we elected to use induced tears for our studies. Human tears had relatively high hemagglutination inhibition titers (geometric mean titer [GMT] of >300) against all viruses tested except NL/230. Overall, titers of NL/219 as well as the two other ocular viruses were lower than those of the nonocular viruses, and this difference was statistically significant ($P < 0.001$) (Table 2).

To evaluate the effect of tears on virus viability, we employed a plaque reduction assay. The virus was mixed with an equal volume of either phosphate-buffered saline (PBS) or diluted tears, incubated for 1 h at room temperature, and then plated onto Madin-Darby canine kidney (MDCK) cells for a standard plaque assay. Viral infectivity remaining after incubation was calculated by dividing the number of plaques in each tear-treated well by the number of plaques in the corresponding PBS well. All of the viruses tested were significantly inhibited by tears diluted 20-fold (Fig. 9A, black bars). As a group, the ocular viruses were less inhibited than were the nonocular ones ($P <$ 0.001 whether or not the NL/219 virus was included in the ocular group), although the percentage of the H5N1 virus, Thai/16, that remained infectious after incubation with tears was within the range of those of the ocular viruses. Tears from a second set of donors were less inhibitory overall, but the difference between ocular and respiratory

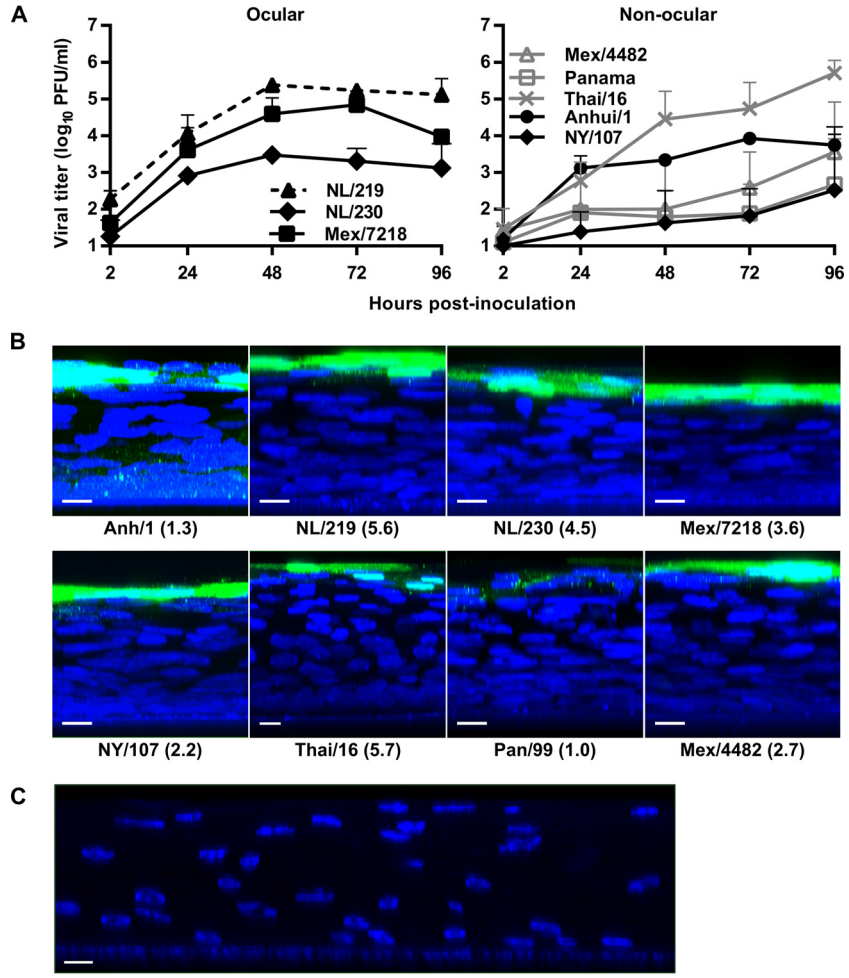

FIG 7 Replication and spread of influenza virus in corneal tissue constructs. Constructs were infected with the indicated viruses at an MOI of 0.01 using traditional liquid-based methods. (A) Curves for NL/230, Anh/1, Thai/16, and Pan/99 viruses are repeated from Fig. 6 for convenience. Points for other viruses represent means and standard deviations of results for duplicate wells. The curve for NL/219 is dashed to indicate that this isolate is unique among the ocular viruses. The limit of detection was 1 $\log_{10}$ PFU/ml. (B) Cells from a second set of constructs were fixed at 96 h postinfection and stained with anti-NP antibody (green) and DAPI (blue). A maximum-intensity projection across the *x* axis is shown. Supernatant titers ($\log_{10}$ PFU/ml) are given in parentheses after the virus names. (C) Nuclear density across a single focal plane. Shown is an orthogonal view of a z-stack of an uninfected construct stained with DAPI. Bars = 10 μm.

viruses was maintained ($P < 0.001$ including NL/219 virus, and $P = 0.004$ excluding NL/219 virus). A dose-response relationship was evident when tears were further diluted, with higher percentages of virions retaining infectivity when mixed with more-diluted tear samples, but this relationship was not identical across viruses, suggesting that different viruses might be primarily inhibited by tear components present in differing concentrations. The difference between ocular and nonocular viruses remained significant at both 1:40 and 1:80 dilutions when NL/219 was excluded from the analysis ($P = 0.004$ and $P = 0.016$, respectively) but only at a 1:40 dilution when NL/219 was included ($P < 0.001$).

In order to investigate which component or components of tears were responsible for the reduction in viral infectivity that we observed, we first tested two antimicrobial proteins present in large quantities in tears: lactoferrin and lysozyme (22). Each protein was substituted for tears in the plaque reduction assay at a concentration equivalent to that expected to be present in the highest concentration of tears used previously (a 1:20 dilution). The viability of all viruses except NL/219 remained high (>75% of the

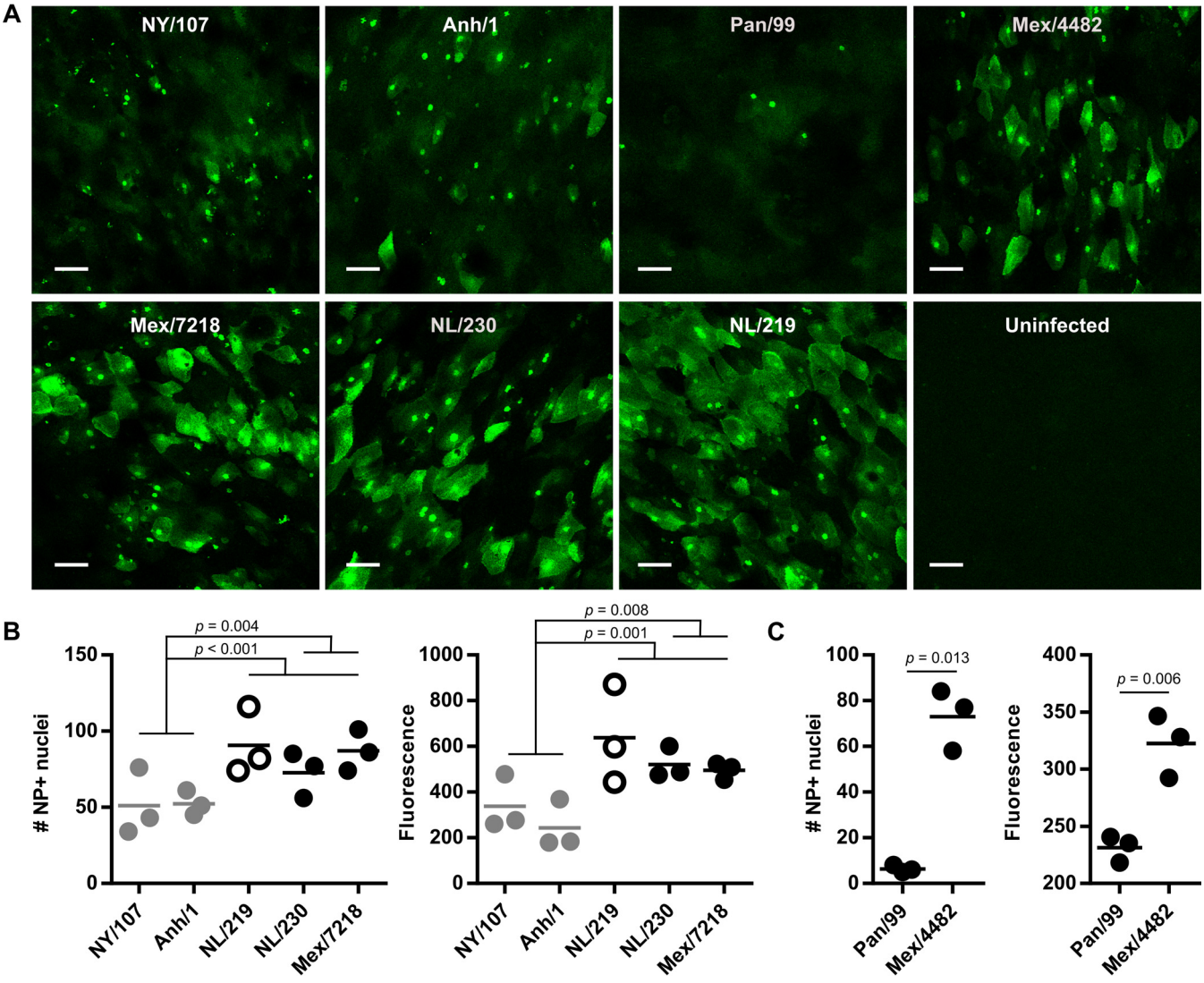

FIG 8 Single-cycle infection of corneal tissue constructs. Corneal constructs were inoculated at an MOI of 2 and then fixed at 8 h postinfection and stained with antibody against the viral nucleoprotein (green). (A) Representative 10× fields. Bars = 100 μm. (B) Number of NP^+ nuclei and fluorescence activity for each of three fields in constructs infected with H7 viruses. Statistical significance was determined by one-way analysis of variance followed by a preplanned posttest performed by using Proc GLM in SAS 9.4. (C) Number of NP^+ nuclei and fluorescence activity per field for each of three fields in constructs infected with human influenza viruses. Statistical significance was determined by a *t* test.

control) after incubation with both proteins, and no difference between ocular and nonocular viruses was evident (Fig. 10). The NL/219 virus was uniquely affected, with only 53% and 39% infectivity being retained after treatment with lactoferrin and lysozyme, respectively.

To evaluate the extent to which the inhibition that we observed was due to the binding of virus to sialic acid present in the tear fluid, we desialylated the tears with exogenous bacterial neuraminidase prior to incubation with virus. Desialylation was confirmed by a reduction of the hemagglutination inhibition (HI) titer to <10. This approach required incubating the tear-neuraminidase mixture at 37°C (to facilitate neuraminidase activity) and then heating the mixture to a higher temperature to inactivate the neuraminidase so that it would not prevent infection by removing the sialic acid receptors from the surface of the MDCK cells used in the plaque reduction assay. However, we found that when it was mixed with the virus after putative inactivation under standard conditions at 56°C for 30 min, neuraminidase alone (in PBS) decreased the number of plaques relative to that seen with PBS only. Increasing the

TABLE 2 Hemagglutination inhibition by human tears

Virus	GMT[a]
Pan/99	403
Thai/16	640
Anh/1	1,016
NY/107	806
NL/219	320
NL/230	10
Mex/7218	403
Mock[b]	<10

[a]GMT, geometric mean titer ($n = 3$).
[b]Normal allantoic fluid.

inactivation temperature to 65°C reduced, but did not eliminate, this effect, the magnitude of which differed by virus (data not shown). We therefore used two controls for each experimental sample: one contained tears but no neuraminidase (to control for the impact of heating on the inhibitory properties of tears), and the other contained neuraminidase but no tears (to control for the residual effect of neuraminidase). Both controls were subjected to the experimental conditions (37°C for 1 h followed by 65°C for 30 min), and the combined effect of the two controls was estimated by adding the individual effects. We then compared the number of infectious particles remaining after incubation with desialylated tears to the number of infectious particles expected from the additive effects of both controls (Fig. 9B). The difference between the two controls, representative of the percentage of PFU inhibited by sialic acid, was greater for ocular (69% including and 72% excluding NL/219 virus) than for nonocular (52%) viruses ($P < 0.001$). In conducting these experiments, we found that heating of tears (37°C for 1 h followed by 30 min at 65°C) reduced their inhibitory activity. This was particularly true for the respiratory viruses, for which the difference in postincubation virus viabilities between heat-treated and non-heat-treated tears averaged 29%, compared to 6% (excluding NL/219 virus) or 7% (including NL/219 virus) for the ocular viruses (Fig. 9C) ($P < 0.001$). These data demonstrate that human tears are highly effective

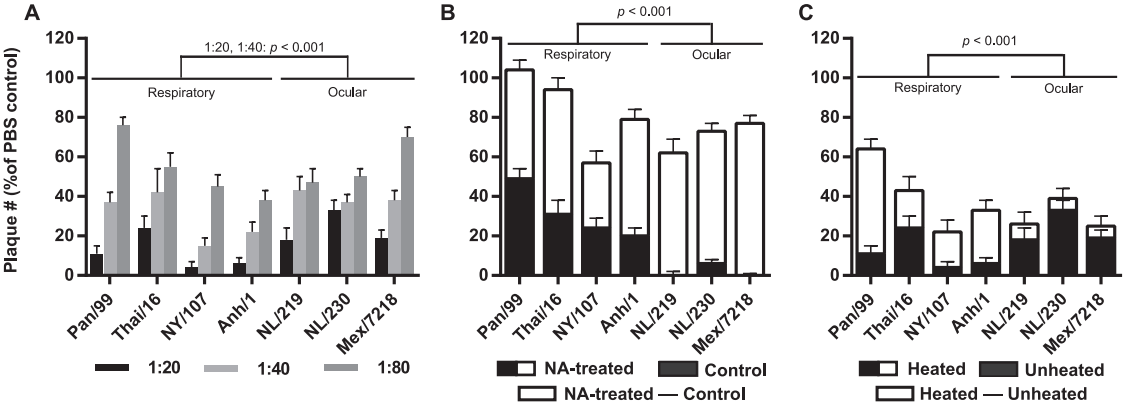

FIG 9 Inhibition of influenza virus by human tears. Virus was mixed with human tears or PBS, incubated at room temperature for 1 h, and then titrated via a plaque assay. Binomial means of data from three experiments plus 95% confidence intervals are shown. Statistical significance was determined via a chi-square test with Bonferroni correction. (A) Human tears reduce virus viability in a dose-dependent manner. The key indicates the final dilution of tears after mixing with virus. (B) Effect of exogenous neuraminidase (NA) treatment on the inhibitory effect of human tears (1:20). The total bar height (black and white bars) indicates the number of plaques for tears that were pretreated with exogenous neuraminidase before mixing with virus. The black region indicates the combined effect of controls. The statistical comparison is for the difference between neuraminidase-pretreated tears and the control (white region). The number of control plaques was determined by subtracting both the number of plaques inhibited by heated tears and the number of plaques inhibited by inactivated neuraminidase from the number of plaques on PBS wells. (C) Effect of heat treatment on the inhibitory effect of human tears (1:20). Tears were incubated at 37°C for 1 h, followed by 30 min at 65°C. The total bar height (black and white bars) represents the percentage of virus that remained infectious after incubation with heat-treated tears. The black region indicates the unheated control. The statistical comparison is for the difference between the percentage of virus that remained infectious after incubation with heated tears and that after incubation with unheated tears (white region).

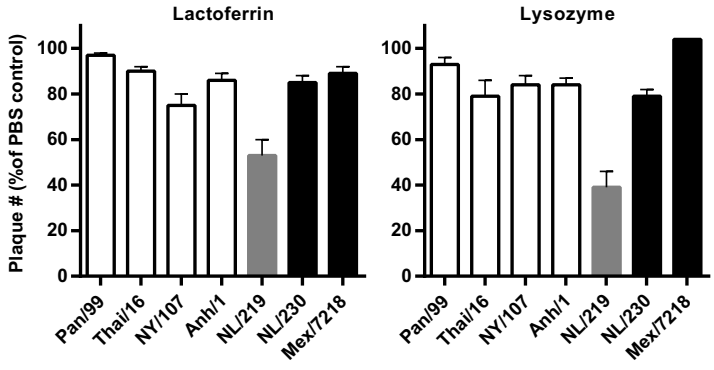

FIG 10 Influenza virus inhibition by human lactoferrin and lysozyme. Lyophilized human lactoferrin or lysozyme was resuspended in PBS at a concentration of 0.15 mg/ml and then mixed with an equal volume of virus. The mixture was incubated at room temperature for 1 h and then titrated via plaque assay. Binomial means of data from three experiments and 95% confidence intervals are shown. White bars, respiratory viruses; black bars, ocular viruses; gray bars indicate that NL/219 virus is unique among the ocular viruses.

in blocking influenza virus infection and suggest that multiple tear components are responsible for this effect.

DISCUSSION

Despite previous studies conducted *in vivo*, *ex vivo*, and *in vitro*, the unique characteristics of influenza viruses associated with ocular infection remain unclear. Whether only a minority of influenza viruses are capable of infecting and replicating in the human eye, or whether ocular involvement is routine but triggers sufficient inflammation to cause symptoms only in select cases, remains an open question, as the eye is not usually sampled in patients with typical influenza-like illness. Previous studies demonstrated the ability of non-conjunctivitis-associated viruses to replicate in primary human ocular cells and *ex vivo* tissues. While this work suggests that cells present on the ocular surface are both susceptible and permissive to infection, it does not necessarily indicate that these viruses are ocular-tropic in humans, where conditions may differ from those used in the laboratory. In this study, we examined the influence of culture temperature, inoculation method, and membrane-associated mucins on the ability of a variety of influenza viruses to infect and replicate within human corneal epithelial cells and investigated the interaction between different influenza viruses and the tear film.

Although ocular aerosol challenge has been used in animals, to our knowledge, this inoculation method has not been used with human ocular cells or tissues. We previously found that primary human alveolar epithelial cells were less susceptible to infection with certain viruses when inoculated via aerosol than when inoculated by more traditional methods (18). To determine whether a similar phenomenon might explain why both seasonal and avian respiratory viruses, previously shown to productively infect multiple ocular cell types (8, 9, 11, 12, 23), rarely establish ocular infection in humans, we inoculated corneal epithelial cells by the aerosol route. All viruses examined proved capable of infecting primary human corneal epithelial cell monolayers after aerosol delivery, suggesting that this is a plausible route of infection but not indicating that aerosol exposure can account for differences in ocular tropism.

In contrast to core body temperature, the temperature of the surface of the eye, like the upper respiratory tract, is approximately 33°C (24, 25). While the relationship between the efficiency of replication in respiratory cells at 33°C and virus transmission has been extensively studied, minimal work on the role of temperature in ocular infection has been performed. Some studies used a culture temperature of 33°C, and others used a temperature of 37°C (8, 9, 11, 12), but a comparison between the two temperatures has not been made. Although ocular isolates, including the two used in this study, typically do not have markers (e.g., PB2 627K or 701N) associated with

enhanced replication efficiency at lower temperatures and replicate poorly at 33°C compared to 37°C in respiratory and MDCK cells, we nonetheless tested the hypothesis that ocular influenza viruses might either infect or replicate better than nonocular viruses at 33°C, as this would be consistent with their ability to establish infection at the ocular surface. However, we found that the effect of culture temperature on viral infectivity and replication in corneal cells was similar to that observed in other respiratory cells; further studies evaluating the susceptibility of other cells present on the ocular surface to infectious aerosols, such as the conjunctival epithelium (shown previously to support virus replication with both avian and human viruses at 37°C), are warranted (12).

Membrane-associated mucins serve a critical barrier function in the eye, preventing pathogens from reaching the immune-privileged cornea (26, 27). These heavily glycosylated proteins are thought to entrap influenza virus particularly well, as the viral hemagglutinin binds efficiently to their abundant sialic acid residues (28). Because the primary cell monolayers used in our initial experiments expressed these mucins at very low levels, we conducted a parallel set of experiments using corneal tissue constructs, which are cultured at the air-liquid interface and express high levels of these mucins at the apical surface. Consistent with the hypothesis that mucins are important determinants of ocular tropism (29), we found that ocular-tropic viruses infected corneal tissue constructs, which express high levels of mucins, more efficiently than did their respiratory-tropic counterparts. Additionally, more extensive cytoplasmic staining was evident in constructs infected with ocular viruses than in constructs infected with nonocular ones, indicating that they were further along in the replication cycle (i.e., ribonucleoproteins were being exported from the nucleus) when cells were fixed.

The use of multilayer tissue constructs also allowed us to examine virus spread within the stratified corneal epithelium. We predicted that ocular influenza viruses might be better able than others to spread vertically, either directly between cells or by diffusion into damaged areas, and would therefore infect deeper layers of the epithelium. In fact, both ocular and respiratory viruses were confined to the apical portion of the constructs. The presence of some NP in the basolateral layer of one construct (Anh/1 virus) (Fig. 8B) demonstrates that the anti-NP antibody was able to penetrate deep into the tissue construct, indicating that the lack of positive staining below the apical layer for other viruses reflects the absence of virus and not a limitation of the staining process itself. This limited distribution of viral NP is consistent with patient symptoms. Although ocular infections by influenza virus are typically described as conjunctivitis, symptoms present in some cases, such as pain and sensitivity to light (6, 30), suggest corneal involvement (31, 32). The inability of the virus to spread beyond the superficial squamous epithelial layer likely explains why changes in vision, which are common features of herpes keratitis and sometimes adenoviral keratoconjunctivitis, have not been reported (31, 33). Alternatively, infection may be limited to the conjunctiva, and symptoms result from the spread of inflammation to the cornea. Further investigation of the extent of differentiation of cells in tissue constructs and of factors that limit spread deeper into the corneal epithelium is warranted.

We found that human tears inhibited viral hemagglutination and infection, even when diluted 20-fold or more. As a group, the ocular influenza viruses retained more infectivity than did their nonocular counterparts when incubated with tears. Interestingly, the nonocular H7 viruses were particularly susceptible to inhibition, suggesting that nonhemagglutinin viral proteins, such as neuraminidase, affect the ability of viruses to avoid inactivation before reaching the host cell surface. The tear film includes a wide variety of antimicrobial factors, including secretory immunoglobulin A (IgA), lipocalin, lactoferrin, lysozyme, and mucins. IgA undoubtedly plays an important role in the protection of the eye from numerous pathogens, possibly including seasonal influenza virus, but cannot account for the infrequency of ocular infection by swine- and avian-origin influenza viruses, as antibodies to these viruses are rare in humans. Additionally, IgA is present in cried tears at much lower levels than in basal tears,

making it difficult to study in the absence of tear specimens obtained by capillary (34). We did not examine lipocalin individually, as neither its ability to bind microbial siderophores nor its DNase activity made it a likely candidate for inhibition of an RNA virus (35, 36). In contrast, antiviral effects of lactoferrin have been documented for numerous viruses (37, 38). In the case of influenza virus, Ammendolia and colleagues found that when incubated with multiple viral subtypes prior to cellular inoculation, even very low concentrations of bovine lactoferrin were able to prevent cytopathic effects by binding to the first 18 amino acids of the fusion peptide of the hemagglutinin (39). Although bovine lactoferrin is 69% identical to human lactoferrin at the amino acid level and the two structures are similar (40), it differs from the human protein by nearly 50% across the three loops thought to bind influenza virus hemagglutinin. Our use of human lactoferrin thus likely explains why we observed such a limited effect on virus viability. The infectivity of NL/219 virus, which was substantially reduced by lactoferrin, was probably affected by a different mechanism, as this virus is identical across the relevant portion of the fusion peptide to the other, less-affected, H7 viruses tested. Known for its ability to cleave the peptidoglycan of the bacterial cell wall, lysozyme also reduces virus production by HIV-infected cells and, albeit when denatured, inactivates murine and human noroviruses (41, 42). Its impact on the influenza viruses tested in this study was similar to that of lactoferrin, with only a minimal effect on all viruses tested except NL/219.

Mucins present in the tear film include both membrane-associated mucins that have been proteolytically cleaved or have broken away from the cell surface and the secreted gel-forming mucin MUC5AC, which is produced by the conjunctival, but not the corneal, epithelium (43, 44). As noted above, the many glycans present on ocular mucins can serve as decoy receptors for sialic acid (SA) binding pathogens, interfering with attachment to the ocular surface. In hemagglutination inhibition assays, ocular influenza viruses were less inhibited by tears (lower HI titers) than were nonocular viruses. The fact that hemagglutination inhibition activity was removed by treatment with exogenous neuraminidase strongly suggests that this inhibition was due to SA. However, the plaque reduction assay yielded the opposite result, with the ocular viruses showing greater infectivity differences between neuraminidase-treated and untreated tears (Fig. 9B). This indicates that binding does not necessarily prevent infection; we suspect that the nonocular viruses may have high neuraminidase activity relative to hemagglutinin affinity, making them better able to free themselves from mucins and go on to initiate infection. Alternatively, the effects of inactivated neuraminidase and of tears are not additive, as we assumed, resulting in an underestimation of the number of plaques in control wells and, thus, an overestimation, particularly in the case of viruses more affected by the inactivated exogenous neuraminidase, of the infectivity difference between neuraminidase-treated tears and controls. In any case, the data do not necessarily suggest, as might have been expected, that viruses with a preference for either α2,6- or α2,3-linked SA receptors are more inhibited by SA in tears: in the hemagglutination inhibition assay, titers for Pan/99 virus, which binds α2,6-linked receptors, were closer to those for the α2,3 binding ocular viruses than to those for Thai/16 virus, a member of a clade which strongly favors α2,3-linked SA (45–47). In the plaque reduction assay, Thai/16 virus behaved more similarly to the ocular viruses than did Pan/99. However, NY/107 and Anh/1 viruses, which prefer α2,3-linked SA but displayed limited binding to α2,6-linked SA, did not have an intermediate phenotype but were more inhibited than any of the other viruses (45, 48, 49). Differences in specificity for parts of the glycan structure aside from the sialic acid-galactose linkage may explain these phenotypic differences; while glycan array data have been reported for some of the viruses tested, data on the types of glycans present in tears are not yet available. The fact that the difference between virus viabilities retained after incubation with heated and unheated tears was greater for ocular-tropic than for respiratory-tropic viruses suggests the presence of an inhibitory, heat-labile component of tears to which ocular influenza viruses are more resistant than those that do not display this tropism in humans. The identity of this component remains

unclear; with approximately 500 proteins present in human tears, there are many possible candidates (50).

Although influenza virus-associated conjunctivitis occurs quite rarely, the potential for the eye to serve as a point of entry and possible human adaptation for viruses that are poorly suited to establishing infection in the respiratory tract represents a cause for public health concern. Knowledge of the factors that permit or restrict ocular infection is key to assessing the significance of this risk. Our findings reinforce the importance of examining the extracellular environment as well as the intracellular one in studies of host-virus interactions.

MATERIALS AND METHODS

Viruses. Viruses used in this study are listed in Table 1. Virus stocks were generated in either MDCK cells (Mex/4482 and Bang/5487) or the allantoic cavity of 10-day-old embryonated hens' eggs (all other viruses), as described previously (51, 52). With the exception of the Pan/99 virus (used at passage 6), all viruses were passaged three times or fewer. Nucleic acid sequences of all virus stocks were confirmed to be identical to those found in the sequence database of the Global Initiative on Sharing All Influenza Data (GISAID). HPAI and LPAI viruses were handled under biosafety level 3 containment, including enhancements, as required by the U.S. Department of Agriculture and the Federal Select Agent Program (53).

Cells. Human primary corneal epithelial cells (ATCC) (cryopreserved at passage 1) and nasal epithelial cells (PromoCell) (cryopreserved at passage 2) were grown according to the manufacturers' instructions. Cells were subcultured and grown to confluence on 24-mm membrane inserts (Transwells; Corning) (4.67-cm^2 growth area). EpiCorneal tissue constructs (MatTek Corporation) (0.62-cm^2 growth area) were unpacked immediately upon receipt and preequilibrated overnight before use the next morning. Constructs were maintained at the air-liquid interface according to the manufacturers' directions. Medium for both corneal monolayers and tissue constructs was serum free. Human bronchial epithelial cells (Calu-3; ATCC) were propagated as described previously (54). Due to the limited commercial availability of primary cells, all cells used in each experiment were sourced from the same donor. Donors of corneal cell monolayers varied across experiments, but all corneal constructs were derived from a single donor, although cells were expanded and differentiated at different times.

Cell infections. MOIs for corneal constructs were calculated based on the midpoint (75,000 cells) of the manufacturer's estimate of 50,000 to 100,000 cells on the apical surface (MatTek). Aerosol inoculations were performed as described previously (18). Briefly, after the removal of apical medium, if present, cells were placed into an aerosol exposure chamber for a 15-min exposure to aerosolized virus, followed by 5 min of room air to purge viral aerosols from the system. For liquid inoculations, apical medium was removed from cell monolayers, and 150 μl (constructs) or 300 μl (monolayers) of medium containing the specified amount of virus was incubated atop the apical surface at 37°C for 1 h, after which the inoculum was removed. Cell monolayers were washed with PBS, and the apical medium of cell monolayers was restored, with the addition of 1 μg/ml *N-p*-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated trypsin (Sigma-Aldrich). Trypsin was not added to the tissue constructs, for which apical medium was not present. At the indicated time points, 200 μl of the supernatant (incubated atop tissue constructs for 20 min before collection) was collected, and an equal volume of fresh, trypsin-containing medium was added to the monolayers. Samples were frozen at −80°C until titration by standard plaque assay in MDCK cells.

Hemagglutination inhibition and infectivity reduction assays. Human tears, pooled from healthy donors, were obtained from Lee BioSolutions, and, except where noted, a single lot was used for all experiments. Tears were induced reflexively or through emotions and collected from the face. Turkey red blood cells (0.5%) were used for all hemagglutination inhibition assays. Since we were specifically interested in the inhibitory effect of sialic acid hemagglutination, tears were not pretreated with receptor-destroying enzyme. Similarly, adsorption of nonspecific agglutinins was not performed, as there was no evidence of agglutination when tears alone were mixed with red blood cells.

For plaque reduction assays, tears were diluted 10-fold, 20-fold, or 40-fold in PBS; mixed with an equal volume of virus diluted to between approximately 60 and 200 PFU/100 μl; and incubated at room temperature for 1 h, after which the mixture was used to inoculate MDCK cells for a standard plaque assay. Virus mixed with PBS, also incubated for 1 h at room temperature, was used for control wells. For each virus, numbers of plaques were summed across three independent assays, and the percentage of infectivity remaining was calculated by dividing the total number of plaques in experimental wells by the total number in control wells. Sialidase pretreatment was performed by adding *Vibrio cholerae* neuraminidase (Sigma-Aldrich) to tears (experimental wells) or PBS (control wells), yielding a final concentration of 0.02 U/ml, and the mixture was incubated at 37°C for 1 h. This treatment was confirmed to reduce the hemagglutination inhibition titer of tears to $<$10, indicating that sialic acids were successfully removed. For this set of experiments only, PBS with calcium and magnesium was used as a control and as a diluent for the tears because calcium is a required cofactor for *V. cholerae* neuraminidase. Lyophilized human milk lactoferrin (Athens Research and Technology) and recombinant human lysozyme (Sigma-Aldrich) were resuspended in PBS and used at a final concentration of 0.15 mg/ml, equivalent to that expected to be present in a 10-fold dilution of human tears (22, 55–58). Count data from the plaque reduction assay were poststratified by using the R survey package (59, 60) such that each virus contributed equally to the result in spite of differences between viruses in the numbers of plaques on

control plates. Comparisons were made by a chi-square test. Binomial confidence intervals for individual viruses were calculated by using the binom package (61).

Immunofluorescence. Cells were washed with PBS and fixed by using 3% paraformaldehyde. Lectin costaining was performed by using fluorescein-conjugated SNA and biotinylated MAA-I plus avidin D-conjugated rhodamine (Vector Laboratories). Additional staining was conducted by using primary antibodies against MUC1 (Cell Signaling Technology, Inc.), MUC4, MUC16, ZO-1 (ThermoFisher Scientific), and influenza virus nucleoprotein (International Reagent Resource) and an Alexa Fluor 488- or 549-conjugated secondary antibody. Cells were mounted with SlowFade Gold (Invitrogen) and imaged with a Zeiss LSM710 confocal microscope.

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